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ABSTRACT

In this article we describe an in vitro model for complement-dependent phagocytosis of liposomes. We have previously reported that complement-opsonized liposomes are avidly ingested by murine peritoneal or bone marrow-derived cultured macrophages. However, when the liposomes contained certain lipids, including phosphatidylinositol, ganglioside $G_{M1}$, and sulfogalactosyl ceramide, that have been identified as causing prolonged circulation time in vivo, complement-dependent phagocytosis of the liposomes was greatly suppressed. We identify certain additional factors associated with suppressed complement-dependent phagocytosis, including liposomal negative charge and liposomal prostaglandin $E_1$ or thromboxane $B_2$. Possible mechanisms responsible for suppression of complement-dependent phagocytosis are suggested. We propose that suppression of complement-dependent phagocytosis could be a contributing factor in the promotion of increased circulation time of "stealth" liposomes and that complement opsonization probably plays a role in vivo in removing liposomes from the circulation.

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# *Liposomes as Safe Carriers of Drugs and Vaccines*

*Carl R. Alving*

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## *Introduction*

Since 1974, as reported by numerous laboratories, hundreds of individual humans have received injections of liposomes either for diagnostic or biodistribution studies or for therapeutic applications as drug carriers (reviewed in [43]). In most studies, the liposomes were injected i.v. in large concentrations, and to date no major difficulties have been reported. These clinical trials were undertaken after extensive previous testing was performed by numerous laboratories, as noted below. The purpose of many in vitro tests and in vivo studies in animals has been to try to ascertain the most likely types of toxicity that might be anticipated in humans.

It is well-known that parenteral injection of multilamellar liposomes into animals results in a massive uptake of the liposomes by the macrophages in the liver, spleen, and bone marrow [37]. The attraction of liposomes to macrophages has been used as the basis of a variety of medical applications of liposomes as carriers of drugs or vaccines [5, 6]. Although beneficial medical applications of liposomes can be demonstrated, the question naturally arises whether the administration of liposomes in therapeutic doses could also cause adverse effects.

## *Reticuloendothelial Blockade*

Detailed acute toxicity studies in mice and beagle dogs failed to demonstrate significant hematological, biochemical, immunological, or histopathological toxicity associated with as many as 6 injections of liposomes over a 2-week period [20]. On the other hand, parenteral administration of empty liposomes can apparently cause transient reticuloendothelial (RE) blockage in mice [2, 15, 17, 21, 30], and histopathological changes in liver and spleen have been reported [3, 41].

In humans, the major consequence of RE blockade is a "spillover" phenomenon in which endotoxin (i.e., lipid A) that would normally be detoxified by RE cells travels into other tissues and organs [9, 26, 32]. Endotoxin spillover can result in numerous clinical sequelae, including acute glomerulonephritis, disseminated intravascular coagulation, neutropenia, pyrogeni-

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city, and shock [9, 26, 32]. If the RE system of mice were frequently or chronically challenged by the experimental administration of liposomes or liposomes containing lipid A, it is reasonable to suppose that long-term consequences caused by the liposomes or endotoxin might be demonstrated. However, a detailed study showed that life-long repeated administration of liposomes, or liposomes containing lipid A, did not result in a decreased longevity of the mice, and no identifiable histopathological effects due to experimental lipid A or liposomes were observed [35].

### *Toxic Effects on Cultured Cells and In Vivo Toxicity of Individual Lipids*

Despite the apparent low level of acute toxicity of liposomes in animals, anecdotal descriptions of various effects of certain liposomes on tissue culture cells and in experimental animals that might be interpreted as suggesting some potential for toxicity have been reported. The treatment of cultured tumor cells, fibroblasts, or erythrocytes with liposomes containing stearylamine caused a loss of viability or hemolysis of the cells [11, 23, 33, 42]. Some inhibitory effects on the uptake of [$^3$H]thymidine were also observed with dicetyl phosphate and phosphatidylserine, but not with phosphatidic acid [23]. Stearylamine also caused granuloma formation after s.c. injection in rabbits [22]. After direct intracerebral injection in mice, liposomes containing stearylamine or dicetyl phosphate caused seizures and death, while liposomes containing phosphatidic acid or lacking a charge produced minimal toxic effects [1]. Liposomes containing phosphatidylserine reportedly caused a variety of neurochemical changes after i.v. administration in rodents [10, 12, 24, 38]. Where they were examined, other liposomes such as neutral phosphatidylcholine liposomes or liposomes containing phosphatidylinositol did not exert similar effects [10].

### *Long-Term Effects of Parenteral Injection in Mice*

When the above studies are taken together along with reported granulomatous changes in the liver and spleen in rodents [3, 41], it is reasonable to question whether the long-term experimental administration of liposomes in rodents might exert previously unrecognized adverse effects. In view of this, it is noteworthy that after the lifelong administration of liposomes in mice, granulomatous reactions in the liver or spleen were not observed [35]. After a lifelong injection treatment with lipid A, liposomes, or liposomes containing lipid A, no significant effects were found on the longevity of BALB/c mice, and no unique histopathological changes were found after more than 2 years of regular injections. An increased susceptibility to infections and enhancement of the metastatic spread of tumors that have been predicted as possible sequelae of liposome injection [4] were not observed.

### *Intravenous Administration of Liposomes in Humans*

As pointed out by Zonneveld and Crommelin [43], some, but not all, studies with humans have reported side effects associated with the injection of liposome-encapsulated drugs. In no case did the side effects alter the treatment, and in only one case, a pyrogenic reaction, was treatment for a side effect reported. In each case in which reactions were observed, the liposomes either contained a cytotoxic drug or the drug was thought to be contaminated with a pyrogenic contaminant, and the drug or contaminant were thought to be the offending agent. Among the reported symptoms in individual studies were headache, mild sleepiness or sedation (reported in two studies), nausea, difficulty in concentration, fever, chills, lumbar pain, urticarial rash, bronchospasm, and leukocytopenia [43].

A recent single case report described acute transient cardiopulmonary toxicity after the daily i.v. infusion of a liposomal and lipid-complex delivery system containing amphotericin for the treatment of hepatic *Candida* abscesses in a patient with stage 4 lymphoblastic lymphoma [25]. In this reaction, reduced cardiopulmonary symptoms were achieved by lowering the doses of the subsequently administered liposomal/lipid complex amphotericin B, thus allowing a satisfactory continuation of therapy.

### *Complement Activation due to Naturally Occurring Antibodies to Liposomes*

In experimental studies with sera from humans, a widespread natural occurrence was observed of IgG and IgM (but not IgE) antibodies to phospholipids [7], cholesterol [8], or lipid A [29], each of which can be major component of liposomes. Complement (C) activation occurs when liposomes are injected intravenously into animals that have such antibodies (such as pigs), and a "pseudoallergic" reaction may occur. My colleagues and I observed a severe anaphylactoid reaction associated with acute hypertension and pulmonary vascular insufficiency that occurred in pigs after the i.v. injection of liposomes [40]. The main cause of this reaction was C activation, with the generation of anaphylatoxin (especially C5a) resulting in the secretion of thromboxane ($TXB_2$), which then caused pulmonary and peripheral vascular hypertension. Secretion of $TXB_2$ leading to respiratory distress has also been reported to occur after the injection of liposomes in sheep [31].

Most normal human sera have antibodies that might react with liposomes to cause C activation. Indeed, C activation was reported in one study in which liposomes were injected into humans [13]. However, it is unlikely that this will pose a significant hazard in the use of liposomes as carriers for drugs or vaccines. This conclusion is based on a variety of evidence. First, if C activation were a substantial problem, i.v. contact of liposomes with plasma would maximize those problems. However, in most studies using liposomes

i.v. in humans, no side effects at all have been reported, or none that cannot be attributed to the liposome-encapsulated drug [27]. Second, in nonliposomal situations in which C activation often occurs, such as renal dialysis (due to dialysis tubing), severe side effects other than the frequent occurrence of transient neutropenia are unusual [14, 19]. In the one reported instance of C activation after the infusion of liposomes, it was concluded that "the activation of the complement system was not associated with clinical manifestations" [13]. Third, it is well-known that humans are much less susceptible than many animals (such as pigs or sheep) to the physiological effects of anaphylatoxins derived from C. For example, it has been reported that human C5a is 10000-fold less spasmogenic than pig C5a [28]. Fourth, symptoms due to C activation in renal dialysis are related to the degree and rate of C activation [14], and under most circumstances i.v. administration can be done slowly; in addition, it is expected that the intramuscular administration of liposomes would cause a slow release of liposomes into the circulation. Fifth, the amounts of i.v. liposomal phospholipids used in studies where symptoms were not observed [27] were very high.

In the single case in which cardiopulmonary toxicity was observed in a compromised patient after the i.v. injection of a drug-containing formulation, the toxicity that was observed could have been compatible with C activation [25]. It is noteworthy that the intensity of symptoms was markedly dependent on the dose of the injected formulation.

### *Liposomal Lipid A as an Intramuscular Vaccine*

Lipid A is the active endotoxic component of bacterial lipopolysaccharide. By itself, it has considerable potential for causing side reactions ranging from pyrogenic reactions to hypotension and death. In a phase I safety trial, monophosphoryl lipid A (MPL) given i.v. was considered safe at 100 $\mu g/m^2$ but not at 250 $\mu g/m^2$ [39]. In contrast, in a phase I safety trial of a recently completed liposomal malaria vaccine containing MPL, the maximum dose of MPL given i.m. was approximately 1200 $\mu g/m^2$, and no significant adverse reaction attributable to liposomes or liposomal MPL was observed even at such high concentrations of MPL [18]. When lipid A is incorporated into liposomes, toxic reactions are greatly diminished. This is because much of the toxic effect of lipid A is caused by the fatty acids of lipid A reacting with cells or tissues, and when lipid A is in liposomes the fatty acids are deeply buried in the lipid bilayer of liposomes.

Previous studies have shown that the reactivity of lipid A in the *Limulus* amebocyte lysate (LAL) assay is eliminated by the incorporation of lipid A in liposomes [16, 34, 36]. In fact, the *Limulus* amebocyte lysate activity was diminished as much as 10000-fold by liposomes [36]. Other potentially toxic reactions of lipid A eliminated by liposomes include leukopenia [36] and the secretion of interleukin-1 [16]. In preclinical experiments leading to the above vaccine study, it was found that rabbit pyrogenicity was reduced 25-

fold to 214-fold by the incorporation of lipid A into liposomes. The liposomal vaccine was nonpyrogenic in rabbits at the dose used in humans, and the vaccine was also essentially nonpyrogenic in humans [18].

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